ANTICANCER RESEARCH *24*: 2249-2256 (2004)

Chemoimmunotherapy of Murine Mammary Adenocarcinomas

IRA H. AMES

Department of Cell and Developmental Biology, State University of New York, Upstate Medical University, Syracuse, New York, U.S.A.

Abstract. *Breast cancer is the most common form of noncutaneous malignancy in the United States. Since a plateau seems to have been reached in the efficacy of conventional therapy for this, as well as for other solid tumors, our laboratory has been studying the therapeutic potential of chemoimmunotherapy against the disease. Here we report on attempts to treat autochthonous mammary adenocarcinomas in C_3H mice with tumor-infiltrating lymphocytes (TILs), interleukin-2 (IL-2) and cyclophosphamide (Cy) alone or in combination. Treatment with unfractionated TILs, IL-2 or Cy reduced tumor growth by 60-70%, when used alone. It was necessary to administer TILs and IL-2 by means of intratumor injection for them to be effective. IL-2 coupled with Cy given intraperitoneally (i.p.) reduced tumor growth by 85%. Adherent TILs (A-TILs) given together with Cy reduced tumor growth to the same extent. It can be concluded from this work that the growth of MMTV-induced mammary tumors in C_3H mice can be significantly inhibited by chemoimmunotherapy. The two most effective protocols were combining Cy with either A-TILs or IL-2. No adverse side-effects were observed in animals that received IL-2 via intratumor injection.*

Since we have reached a plateau in the efficacy of conventional therapy for solid malignancies, a number of novel therapeutic options are being studied. The engagement of immune defense is one such option. An example of this approach is adoptive immunotherapy (AI), or cell-transfer therapy, which involves transfer of cells with antitumor activity to a tumor-bearing host. AI has been attempted with several populations of lymphocytes with varying degrees of success. One such population consists of lymphocytes that infiltrate into growing cancers and can be expanded *in vitro*. These tumor-infiltrating lymphocytes (TILs) are more effective *in vitro* than lymphokine-activated killer (LAK) cells (1), and it has been found that they concentrate at tumor sites more than in corresponding normal tissue following adoptive transfer (2-4). In the first clinical trial of TILs, four out of six patients receiving multiple intravenous or intralesional injections of cells cultured from biopsy specimens showed tumor regression (5). Rosenberg's group observed several partial responses to therapy by nine patients with advanced cancer treated with TILs, interleukin-2 (IL-2) and cyclophosphamide (Cy) (6). They also reported that 38% of patients with metastatic melanoma treated by this approach had objective evidence of cancer regression (7). Kradin *et al.* (8) achieved tumor reduction in some patients with advanced malignant melanoma and renal cell carcinoma by AI with TILs and IL-2, while Ratto *et al.* (9) noted a positive therapeutic response in the postoperative treatment of resected non-small cell lung carcinoma. It has also been reported that AI with TILs achieves high response rates even without IL-2 administration in patients with epithelial ovarian cancer (10). Kono *et al.* (11) observed that AI with TILs in combination with chemotherapy was effective in prolonging survival in cases of gastric cancer. Nevertheless, interest in AI of solid tumors with TILs has waned during the last few years, because the protocol has yielded limited positive results. However, the results of two recent clinical studies using AI with melanoma-specific T cells have breathed new life into this form of cancer treatment. Dudley *et al.* (12) reported objective clinical responses in six out of thirteen melanoma patients treated with T cells derived from TILs and high-dose IL-2 following immunodepleting chemotherapy. In a phase I study of AI using tumor-reactive $CD8^+$ T cell clones generated from patients with metastatic melanoma and relatively low doses of IL-2, Yee *et al.* (13) observed stabilization of disease in 50% of patients and minor or mixed responses in three additional patients with advanced disease.

Breast cancer, one of the major causes of death in industrialized nations, is the most common type of noncutaneous malignancy in the United States. More than 180,000 women in this country are diagnosed with the disease every year. In spite of these alarming statistics, few published attempts have been made to treat mammary adenocarcinoma by means of AI. In an early progress report,

Correspondence to: Dr. Ira H. Ames, Department of Cell and Developmental Biology, SUNY Upstate Medical University, 766 Irving Avenue, Syracuse, New York 13210, U.S.A.

Key Words: Chemoimmunotherapy, mouse mammary tumors.

Rosenberg (14) described treating only two breast cancer patients with LAK cells plus IL-2, with no positive responses. West *et al.* (15) included one woman with carcinoma of the breast in their study of AI with LAK cells and constant infusion of IL-2. This patient also failed to respond to therapy. A single breast cancer case, included in a pilot study published by Rosenberg's group (6), experienced a partial regression of disease in lymph nodal and cutaneous sites with complete elimination of malignant cells from a pleural effusion after treatment with TILs, IL-2 and Cy.

Since combined modality therapy is being utilized more frequently in the treatment of a wide variety of cancers, a major goal of this laboratory has been to determine the therapeutic potential of chemoimmunotherapy against mammary adenocarcinomas. We have chosen to work with C_3H mice which carry mouse mammary tumor virus (MMTV) and have a high incidence of mammary cancer. While it may be more convenient to work with transplantable mammary tumors, we agree with several others that autochthonous mouse tumors are more appropriate models of human cancer (16,17). We have developed protocols for isolation and expansion of TILs from such tumors. The morphology, cell surface phenotype, *in vitro* functional activity and preferential homing patterns of these cells have been described (2,18).

Here we report the results of treating C_3H mouse mammary adenocarcinomas with TILs, IL-2 and Cy alone or in combination. IL-2, a 15-kd glycoprotein produced by helper T-lymphocytes, acts as a growth factor for lymphocytes and increases their *in vitro* cytotoxic potential against autologous tumor cells (19). Cyclophosphamide is an alkylating agent frequently used in cancer chemotherapy and treatment of autoimmune disease. It has recently been shown to have a high *in vitro* response rate when tested against tumor-derived breast carcinoma cells (20).

Materials and Methods

Animals. Some of the preliminary experiments utilized mice of the C_3H/HeOuJ strain purchased as retired breeder females from the Jackson Laboratory, Bar Harbor, ME, USA. Most of the work was done with C_3H/HeN mice purchased at 6-8 weeks of age from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD, USA. All mice were healthy upon arrival and remained disease-free for the duration of the experiments. They were maintained in the Department of Laboratory Animal Resources at the SUNY-Upstate Medical University in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services and the National Institutes of Health. Beginning at 20 weeks of age, all mice were checked for palpable mammary tumors once a week. Institutional guidelines were followed for the end point of tumor studies.

Isolation of tumor-infiltrating lymphocytes. Mammary adenocarcinomas were excised aseptically from euthanized female mice. They were rinsed with RPMI 1640 medium supplemented with 10% fetal bovine serum (RPMI-FBS), and surrounding connective tissue, necrotic tissue and blood clots were removed. The remaining tissue was minced with scissors in a petri dish and forced through a stainless-steel wire mesh. The resulting cell suspension was allowed to sediment for 40 min. The supernatant was removed, passed through 44-μm nylon mesh and centrifuged at 300 g for 15 min. Cells were resuspended in RPMI-FBS, layered over Lympholyte M (Cederlane Laboratories, Hornby, Ontario, Canada) and centrifuged at 550 g for 30 min. Cells at the interface between layers were harvested and washed three times in RPMI 1640 medium.

Isolation of mammary tumor cells. Primary mammary tumors were obtained from euthanized female mice. They were excised aseptically and rinsed with Hank's balanced salt solution (HBSS). After the removal of blood clots and necrotic areas, the tumor tissue was minced with scissors. Cell suspensions were prepared by treatment of the finely minced tumor tissue with collagenase (297 units/ml), trypsin (2 mg/ml) and DNAase (0.2 mg/ml) in HBSS with 10 mM Hepes buffer adjusted to pH 7.3 for 2.5 h at 37˚C in a shaking water bath. The resulting cell suspension was passed through stainless-steel wire mesh followed by 80-μm nylon mesh, and the cells were collected by centrifugation, washed and resuspended in 5.0 ml RPMI-FBS. Cell separation was achieved by centrifugation through a discontinuous gradient composed of 2.0 ml layers of 66.7%, 40.8%, 35.4%, 30.0% and 20.0% Percoll (Pharmacia, Uppsala, Sweden). Cells were collected from the interface between 30.0% and 35.4% Percoll, washed twice with RPMI-FBS and resuspended in 1.0 ml RPMI containing 20% FBS. Mammary tumor cells were frozen in medium containing 15% FBS and DMSO and kept under liquid N_2 until required. At that time they were thawed, resuspended in complete medium (CM) consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.03% glutamine, 0.10 mM nonessential amino acids, 1.0 mM sodium pyruvate, $5X10^{-5}$ M 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μg/ml) and gentamicin (40 μg/ml), and allowed to recover overnight at 37˚C in a humidified 5% CO_2 atmosphere. They were then irradiated (5,000 rads) in a Gammacell 1000 cell irradiator equipped with a cesium 137 source.

Expansion of tumor-infiltrating lymphocytes. After the third wash in RPMI 1640 medium, TILs were resuspended in CM at a density of $2.5X10^5$ cells/ml. rIL-2 was added to achieve a final concentration of 500U/ml. Mixed cultures of fresh TILs and irradiated mammary tumor cells at a lymphocyte/tumor cell ratio of 10:1 were established in 24-well culture plates (2.0 ml/well) at 37˚C in a humidified 5% CO_2 atmosphere. After 3 days in culture, the cells were refed with CM containing rIL-2 (500U/ml). On day 5 the cells were refed with CM containing rIL-2 (500U/ml) and irradiated mammary tumor cells at a density of $0.375X10^5$ cells/ml. One week later the cells were refed with CM containing rIL-2 (500U/ml), and this refeeding schedule was continued for 4 additional weeks. At that time the cells were harvested, collected by centrifugation, counted, resuspended at $1.0X10^6$ cells/ml in fresh CM containing rIL-2 (500U/ml), and pipetted into 25-cm^2 tissue culture flasks. During extended periods of growth in culture, cells were passaged every 7 days.

Generation of adherent and nonadherent TILs. At the time of passage, nonadherent cells were removed, and the flasks were vigorously washed with 10 ml of prewarmed (37˚C) CM to remove cells attached to the plastic. Both populations of cells were adjusted to $5.0X10^5$ cells/ml and cultured for 1 week. This protocol was followed until 2 distinct populations of cells were generated. One of these did not adhere to the tissue culture plastic, while the other lightly adhered. From this point the two populations of TILs were cultured separately in CM containing rIL-2 (500U/ml), and the time between passages was reduced to 3-4 days.

Evaluation of cell morphology and viability. The morphology of all cell preparations was evaluated by microscopic analysis of cytocentrifuge preparations. The viability of these preparations was determined by the trypan blue exclusion test, which in all cases was greater than 90%.

Adoptive transfer of TILs. One of the advantages of the C_3H mouse mammary tumor system is that tumors can be easily detected by palpation and their size measured with vernier calipers. All treatment began when the tumor volume reached approximately $500mm^3$. At that time mice were randomly assigned to control or experimental groups. For some preliminary experiments, TILs were injected into the lateral tail vein. However, this route of administration was found to be ineffective (data not shown). This was probably due to the rapid destruction of effector cells upon first passage through the lungs, liver and spleen and the subsequent failure of sufficient numbers of TILs to reach the tumors. Consequently the work reported here was done with locally administered cells.

On day 0, $10.0X10^6$-$15.0X10^6$ cells suspended in 0.2ml RPMI 1640 medium were injected in a split dose directly into the tumors. In the first set of experiments preparations of total TILs were employed, while in the second set adherent TILs were used, since they appeared to be more effective. Control mice received a split dose of 0.2ml RPMI 1640 medium injected intratumorally. This treatment regimen was repeated on days 7 and 14. In order to determine the efficacy of therapy, tumor size was measured with calipers once a week for 3 weeks. It was not possible to extend experiments beyond 21 days, since the growth of untreated and control tumors was so rapid that they had frequently exceeded our institutional guidelines for end point of tumor studies by that time. Tumor volumes were calculated by means of the formula:

$$V=\left[\frac{\pi}{6}\right]d^2D$$

which assumes that the shape of the tumor is that of an ellipsoid of revolution around the long diameter (D).

Animals were euthanized if the tumor size exceeded our institutional guidelines for end point of tumor studies, when there was tumor ulceration, or when there were other indications of animal distress.

IL-2 treatment of tumors. IL-2 was obtained through the NIH AIDS Research and Reference Program, Division of AIDS, NIAID, NIH from Hoffmann-La Roche, Inc. When the lymphokine was used alone, mice received $10X10^3$ units in 0.1ml RPMI 1640 medium as a single intratumor (*i.t.*) injection once a week for three weeks. Control animals were given 0.1ml RPMI medium. Tumors were measured with calipers once a week

Cy treatment of tumors. Cyclophosphamide was obtained from Sigma Chemical Co. (St. Louis, MO, USA). When the drug was used alone, mice received 5.0mg Cy in 0.2ml sterile saline intraperitoneally (*i.p*) once a week for three weeks. Control animals received 0.2ml sterile saline. Tumors were measured with calipers once a week.

Chemoimmunotherapy of tumors. In order to determine whether there was synergy between chemotherapy with Cy and immunotherapy with TILs and/or IL-2, groups of animals were treated with various combinations of the three. The dosage and timing of the treatments with TILs and IL-2 was as described above. Cyclophosphamide was administered 3 days after the mice received IL-2 and/or TILs. Appropriate controls were established for each combined treatment protocol. Experiments ran for 3 weeks and tumors were measured once a week.

Statistical analysis. Treatment groups consisted of at least six mice and all experiments were performed twice with comparable results. Tumor growth data are presented as the mean±SE. The results were evaluated for statistical significance by paired Student's *t*-tests.

Results

Numerous attempts were made to administer TILs systemically by means of tail vein injections to tumor-bearing mice; however, in no case was the rate of tumor growth altered (data not shown). Therefore, in all experiments reported here, TILs were injected directly into tumors. The first series of experiments was done to determine whether treatment with unfractionated TILs, IL-2, or Cy alone would inhibit the growth rate of autochthonous mouse mammary adenocarcinomas. Tumors grew rapidly in the control and untreated animals (Figure 1A) and, by 21 days after treatment had begun, tumor volume had increased by about 12-fold. There was no significant difference in tumor growth rate between controls and untreated animals. On the other hand, injection of at least $10X10^6$ unfractionated TILs into tumors once a week for 3 weeks significantly reduced the rate of tumor growth. This effect was apparent by day 7, and on days 14 and 21 tumor volume was significantly lower by 60-70% in treated mice. Cy alone given *i.p* or intratumor injection of IL-2 were equally effective.

The results of the experiments designed to determine whether there is synergy between chemotherapy with Cy and immunotherapy with unfractionated TILs and /or IL-2 are presented in Figure 1B. Once again tumors grew rapidly in untreated animals, achieving an 8-fold increase in volume by the end of the treatment period. While it may appear that all experimental protocols were effective, statistical analysis revealed that only the combination of intratumor IL-2 and intraperitoneal Cy achieved a significant reduction in the rate of tumor growth. This was greater than that achieved with either treatment alone (Figure 1A).

As shown in Figure 1A, tumor volume in mice that received intratumor injections of unfractionated TILs was significantly

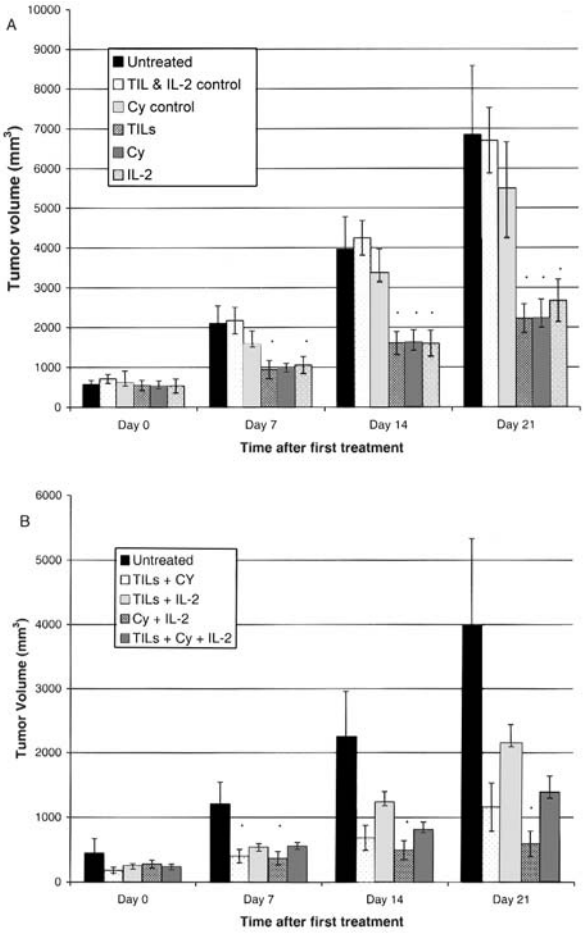

Figure 1. *Inhibition of tumor growth by intratumoral administration of unfractionated TILs and IL-2 and intraperitoneal injection of cyclophosphamide. Tumor volume was determined from weekly caliper measurements. Results are presented as mean ± SE. Treatment groups consisted of at least 6 mice, and all experiments were performed twice with comparable results. Asterisks indicate significant differences ($p<0.05$) between indicated groups and controls. A) Treatment with unfractionated TILs, IL-2, or cyclophosphamide alone. B) Treatment with unfractionated TILs, IL-2 and cyclophosphamide in combination.*

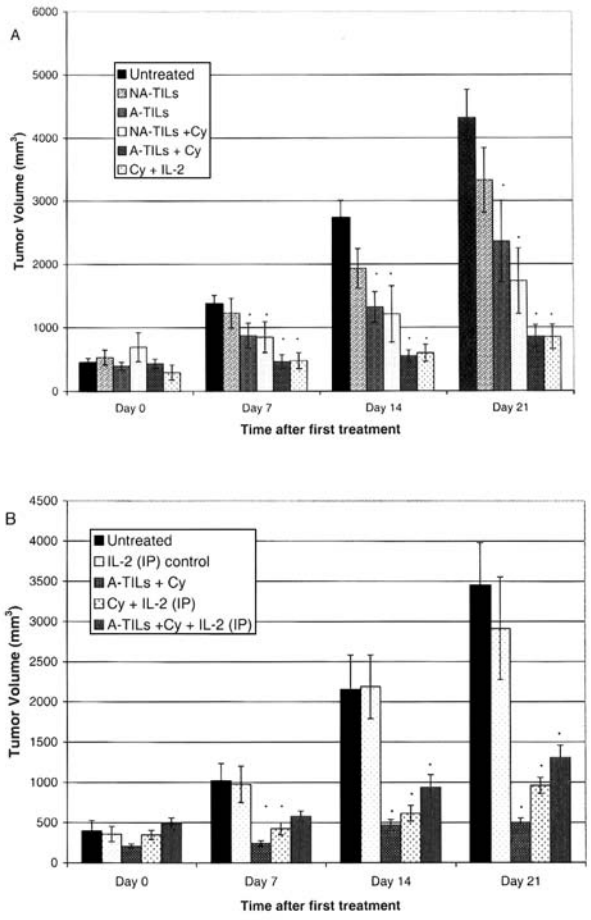

Figure 2. *Inhibition of tumor growth by administration of fractionated TILs, IL-2 and cyclophosphamide. Tumor volume was determined from weekly caliper measurements. Results are presented as mean ± SE. Treatment groups consisted of at least 6 mice, and all experiments were performed twice with comparable results. Asterisks indicate significant differences ($p<0.05$) between indicated groups and controls. A) Treatment with fractionated TILs and IL-2 given i.t. and cyclophosphamide given i.p. B) Treatment with adherent TILs given i.t. and IL-2 and cyclophosphamide given i.p.*

reduced on days 14 and 21. Data comparing the efficacy of treatment with non-adherent and plastic adherent TILs is shown in Figure 2A. As expected tumors in untreated animals grew rapidly. There was a difference in the response of tumors to the two TIL preparations. The growth rate of tumors receiving injections of non-adherent TILs was not significantly different from that of untreated tumors, while that of tumors treated with plastic adherent TILs was significantly reduced. For this reason, adherent TILs were used in all subsequent experiments involving adoptive immunotherapy. As can also be seen in Figure 2A, combined treatment with either adherent TILs and Cy or Cy and IL-2 significantly reduced tumor growth throughout the experimental period, and was more effective than treatment with A-TILs alone on days 14 and 21. However, the results obtained with the two chemoimmunotherapy protocols did not differ from one another at any time point. In both cases, at 3 weeks after the beginning of treatment the volume of treated tumors was about 20% that of tumors in untreated animals.

Combined intratumor treatment with TILs and IL-2 was not tolerated well by the animals. This was due to the fact that this protocol required mice to receive three injections

on the same day. This was a particular problem on day 7, when the tumor size was small. For this reason attempts were made to administer the lymphokine *i.p.* The results of these experiments are presented in Figure 2B. As expected tumor growth in untreated animals was rapid. When IL-2 was given *i.p* it was not effective. This differed from what was observed when it was administered by *i.t.* injection (Figure 1A). However, treatment with Cy and IL-2 (*i.p*) did significantly reduce the rate of tumor growth, but this protocol was not as effective as combining Cy and *i.t.* IL-2 (Figures 1B and 2A).

Intratumor injection of A-TILs combined with Cy given *i.p* significantly reduced tumor growth throughout the experimental period (Figure 2B). On day 21 the mean tumor volume in treated mice was only about 15% that of untreated controls. In this series of experiments, A-TILs given in conjunction with Cy were more effective than the combination of Cy and *i.p.* IL-2. Finally, combined treatment with A-TILs, Cy and IL-2 (*i.p*) was no more effective in reducing tumor growth rate than the protocol utilizing unfractionated TILs, Cy and *i.t.* IL-2 (Figure 1B). On day 21 tumor volume was about 35%-40% of that observed in untreated mice in both cases.

Discussion

Treatment of patients with advanced cancer presents a serious challenge to the biomedical community, because we have reached a plateau in the effectiveness of conventional therapy. In the United States 12% of women will develop breast cancer, and the incidence of the disease is increasing. In spite of this alarming statistic, the clinical emphasis is still on surgery, radiotherapy and chemotherapy, and few new therapeutic modalities are being applied to the treatment of mammary adenocarcinoma. Immunotherapy, which attempts to tip the balance of the immune system toward tumor rejection, may be emerging as a fourth treatment. However, to date it has been shown to have limited efficacy against breast cancer.

A potential approach to this problem is AI, or cell-transfer therapy. Interest in this form of immunotherapy, which was initially high, has diminished over time. This is due to the fact that the protocol is cumbersome to administer and has produced limited positive clinical results. However, the results of several recent studies (12,13) have breathed new life into this form of cancer treatment. They have demonstrated the persistence of adoptively transferred antitumor lymphocytes *in vivo*, their preferential homing to tumor sites, as well as their antitumor effect. For the past few years the focus of this laboratory has been on the study of the efficacy of AI with a variety of cell types, including NK cells, LAK cells and TILs, against autochthonous mouse mammary tumors. We have described the morphology, cell surface phenotype, *in vitro* functional activity and *in vivo* homing patterns of TILs prepared from mouse mammary adenocarcinomas (2,18).

The objective of this investigation was to determine the efficacy of treatment of C_3H mouse mammary adenocarcinomas with TILs, IL-2 and Cy alone or in combination. TILs are a subpopulation of lymphocytes that infiltrate into growing cancers. Rosenberg *et al.* (1) showed that murine TILs are 50-100 times more effective in their therapeutic potency than are lymphokine-activated killer (LAK) cells. Several laboratories, including this one, have shown that they traffic and can localize preferentially to tumor tissue (2-4,12,13). Therefore TILs may be the cells of choice for use in AI of advanced cancer. IL-2 is a growth factor for lymphocytes and increases their *in vitro* cytotoxic potential against autologous tumor cells (19). The cytokine has been approved by the Food and Drug Administration for use in treatment of metastatic renal carcinoma and malignant melanoma. However, when administered systemically in high doses, it has severe side-effects that limit its use. Cy is a chemotherapeutic agent that is used clinically for treatment of cancer and some autoimmune diseases. In spite of the fact that its mechanism of action is not completely understood, it has been used successfully in combination with IL-2 in a number of animal tumor models (21-24).

The results of these experiments demonstrate conclusively that it is possible to significantly inhibit the growth of MMTV-induced mammary tumors in C_3H mice. After three weeks of treatment the tumor volume in mice that received unfractionated TILs, IL-2, or Cy was only 30-40% that of tumors in untreated animals. It is interesting that the efficacy of the three treatment modalities did not differ from one another. It must be noted that it was necessary to administer TILs and IL-2 by means of *i.t.* injection for them to be effective. In several animal cancer models it has been shown that locoregional immunotherapy with IL-2 is more effective than systemic treatment (25-30). This is probably due to the fact that the IL-2 concentration within tumors is higher after *i.t.* injection than following *i.v.* infusion. Locoregional administration of IL-2 also reduces the toxicity of the lymphokine. We observed no evidence of vascular leak syndrome, such as fluid accumulation in tissue spaces and severe hypotension, in mice that received IL-2 *via i.t.* injection. The results of several successful clinical trials of intralesional therapy with IL-2 have been reported (31-34). It was suggested that IL-2 injected *i.t.* acts by enhancing endogenous tumor-specific cytotoxic T lymphocytes and inhibiting tumor-associated vascularity (30).

The success of AI depends upon maximizing the number of effector cells reaching the target. When given by *i.v.* infusion, this is dependent upon their ability to leave the blood stream and enter the tumor. It has been shown that the fraction of TILs given *i.v.* that accumulate in tumors is small (3,35). However, there is some indication from the work of Sacchi *et*

al. (36) that local or perilesional AI with LAK cells and IL-2 is more effective than systemic therapy. Recently, Kjaergaard *et al.* (37) showed that locoregional administration of LAK cells achieved a higher concentration of effector cells at tumor sites than *i.v.* infusion. A second advantage of *i.t.* injection is that it reduces the number of cells that must be generated for each round of therapy. For these reasons, we chose to use *i.t.* injection of TILs in this investigation, since *i.v.* infusion was not effective in the C_3H mouse mammary tumor model. It might be argued that it would be difficult to translate this protocol into the clinical treatment of human breast cancer. However, development of techniques for guided fine-needle aspiration biopsy of nonpalpable breast lesions suggests that it could be done (38,39).

When unfractionated TILs were given in conjunction with Cy or IL-2 (Figure 1B), it appeared that tumor growth was slowed, but the data did not meet the criterion for statistical significance. The same was true for the effect of combining all three agents. However, it is felt that this was due to the wide variability in the growth rate of tumors in untreated control animals and that these results may have biological significance. It is interesting in this regard that, with the exception of the combination of unfractionated TILs and IL-2, the results obtained with the other three protocols did not differ from one another. Nevertheless, *i.t.* injection of IL-2 coupled with Cy given *i.p* did significantly reduce tumor growth to about 15% that of untreated mice. This was a better result than achieved with either agent alone (Figure 1A).

During the course of establishing TILs in culture, we observed that there were two populations of cells. One of these grew in suspension, while the other adhered to the surface of the culture flask. Since it had been shown that natural killer (NK) cells that grew adherent to tissue culture plastic exhibited enhanced antitumor activity (40), we attempted to determine whether the same is true for TILs. It is clear from the data presented in Figure 2A that tumor growth was slowed more by A-TILs than by those that grew in suspension. Curiously, they did not appear to be as effective as unfractionated TILs (Figure 1A). However, when they were given together with Cy, tumor growth was reduced to the same extent as it was with the combination of Cy and IL-2. This was not the case when total TILs were combined with Cy (Figure 1B).

As mentioned above, we experienced difficulty injecting twice into small tumors on the same day. For this reason, attempts were made to administer IL-2 *i.p.*. It can be seen in Figure 2B that such attempts were not successful. This is probably due to the fact that higher concentrations of the lymphokine can be achieved within tumors by means of locoregional application than with systemic treatment. It should be noted that combining Cy with IL-2 given *i.p* was less effective than combining the chemotherapeutic agent with IL-2 given by *i.t.* injection.

It can be concluded from this work that growth of MMTV-induced mammary tumors in C_3H mice can be suppressed up to 80-85% by means of chemoimmunotherapy. It is interesting to note that Nowak *et al.* (41) recently observed that chemotherapy synergized with immunotherapy in the treatment of established murine solid tumors. We found that the two most effective protocols were combining Cy with either A-TILs or IL-2. In both cases Cy was given *i.p*, while the second agent was injected directly into the tumors. At no time during the course of this investigation did we observe complete tumor regression, even in animals treated for up to 6 weeks (data not shown). It is felt that this was due to the size of tumors at the time of initial treatment. It was necessary to delay therapy until the tumor volume reached about 500 mm^3. Lesions were detectable by palpation earlier, but were too small to receive the innoculum. Had we been able to begin treatment when the tumors were smaller, efficacy may have been improved. A similar dependence of therapeutic response on tumor size when treatment began was also observed by Jackaman *et al.* (30).

Acknowledgements

I am grateful to Ms. Nancy Fiore for her assistance throughout this investigation and to Dr. Allen Silverstone for the use of some of his laboratory equipment. The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Interleukin 2 from Dr. Maurice Gately, Hoffmann-La Roche Inc. This research received support from the Education and Research Fund of the Department of Cell and Developmental Biology, State University of New York, U.S.A.

References

1 Rosenberg SA, Spiess P and Lafreniere R: A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science *233*: 1318-1321, 1986.

2 Ames IH, Gagne GM, Garcia AM, John PA, Scatorchia GM, Tomar RH and McAfee JG: Preferential homing of tumor-infiltrating lymphocytes in tumor-bearing mice. Cancer Immunol Immunother *29*: 93-100, 1989.

3 Fisher B, Packard BS, Reed EJ, Carrasquillo JA, Carter CS, Topalian SL, Yang JC, Yolles P, Larson SM and Rosenberg SA: Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. J Clin Oncol *7*: 250-261, 1989.

4 Griffith KD, Read EJ, Carrasquillo JA, Carter CS, Yang JC, Fisher B, Aebersold P, Packard BS, Yu MY and Rosenberg SA: *In vivo* distribution of adoptively transferred indium-111-labeled tumor infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma. J Natl Cancer Inst *81*: 1709-1717, 1989.

5 Kradin RL, Boyle LA, Preffer FI, Callahan RJ, Barlai-Kovach M, Strauss HW, Dubinett S and Kurnick JT: Tumor-derived interleukin-2-dependent lymphocytes in adoptive immunotherapy of lung cancer. Cancer Immunol Immunother *24*: 76-85, 1987.

6 Topalian SL, Solomon D, Avis FP, Chang AE, Freerksen DL, Linehan WM, Lotze MT, Robertson CN, Seipp CA, Simon P, Simpson CG and Rosenberg SA: Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study. J Clin Oncol *6*: 839-853, 1988.
7 Rosenberg SA: Adoptive cellular therapy: Clinical applications. *In*: Biologic Therapy of Cancer. (De Vita VT, Hellman S and Rosenberg SA, eds). Philadelphia, J.B. Lippincott, 1991, pp. 214-236.
8 Kradin RL, Kurnick JT, Lazarus DS, Prefer FI, Dubinett SM, Pinto CE, Gifford J, Davidson E, Grove B, Callahan RJ and Strauss HW: Tumour-infiltrating lymphocytes and interleukin-2 in treatment of advanced cancer. Lancet *1*: 577-580, 1989.
9 Ratto GB, Zino P, Mirabelli S, Minuti P, Aquilina R, Fantino G, Spessa E, Ponte M, Bruzzi P and Melioli G: A randomized trial of adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 *versus* standard therapy in the postoperative treatment of resected non small cell lung carcinoma. Cancer *78*: 244-251, 1996.
10 Aoki Y, Takakuwa K, Kodama S, Tanaka K, Takahashi M, Tokunaga A and Takahashi T: Use of adoptive transfer of tumor-infiltrating lymphocytes alone or in combination with cisplatin-containing chemotherapy in patients with epithelial ovarian cancer. Cancer Res *51*: 1934-1939, 1991.
11 Kono K, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, Sekikawa T and Matsumoto Y: Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. Clin Cancer Res *8*: 1767-1771, 2002.
12 Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE and Rosenberg SA: Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science *298*: 850-854, 2002.
13 Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E and Greenberg PD: Adoptive T cell therapy using antigen-specific $CD8^+$ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci *99*: 16168-16173, 2002.
14 Rosenberg SA: Immunotherapy of cancer using interleukin 2: current status and future prospects. Immunol Today *9*: 58-62, 1988.
15 West WH, Tauer KW, Yannelli JR, Marshall GD, Orr DW, Thurman GB and Oldham RK: Constant-infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. N Engl J Med *316*: 898-905, 1987.
16 Lee JC, Kim DC, Gee MS, Saunders HM, Sehgal CM, Feldman MD, Ross SR and Lee WMF: Interleukin-12 inhibits angiogenesis and growth of transplanted but not *in situ* mouse mammary tumor virus-induced mammary carcinomas. Cancer Res *62*: 747-755, 2002.
17 Wigginton JM, Park J-W, Gruys ME, Young HA, Jorcyk CL, Back TC, Brunda MJ, Strieter RM, Ward J, Green JE and Wiltrout RH: Complete regression of established spontaneous mammary carcinoma and the therapeutic prevention of genetically programmed neoplastic transition by IL-12/pulse IL-2: induction of local T cell infiltration, Fas/Fas ligand gene expression, and mammary epithelial apoptosis. J Immunol *166*: 1156-1168, 2001.
18 Ames IH, Gagne GM, Weiner DL and Tice DG: Characterization of tumor-infiltrating lymphocytes from murine mammary adenocarcinomas. Anticancer Res *14*: 881-888, 1994.
19 Itoh K, Tilden AB and Balch CM: Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. Cancer Res *46*: 3011-3017, 1986.
20 Kornblith P, Wells A, Gabrin MJ, Piwowar J, George LD, Ochs RL and Burholt D: Breast cancer-response rates to chemotherapeutic agents studied *in vitro*. Anticancer Res *23*: 3405-3412, 2003.
21 Stewart LS, Sewell HF and Thomson AW: Combination chemo-immunotherapy: kinetics of *in vivo* and *in vitro* generation of natural killer cells and lymphokine-activated killer cells in the rat. Clin Exp Immunol *79*: 416-423, 1990.
22 Papa MZ, Yang JC, Vetto JT, Shiloni E, Eisenthal A and Rosenberg SA: Combined effects of chemotherapy and interleukin 2 in the therapy of mice with advanced pulmonary tumors. Cancer Res *48*: 122-129, 1988.
23 Lee K, O'Donnell RW, Marquis D and Cockett AT: Eradication of palpable intradermal murine bladder tumors by systemic interleukin-2 and cyclophosphamide in C_3H mice. J Biol Response Mod *7*: 32-42, 1988.
24 Silagi S and Schaefer AE: Successful immunotherapy of mouse melanoma and sarcoma with recombinant interleukin-2 and cyclophosphamide. J Biol Response Mod *5*: 411-422, 1986.
25 Keilholz U, Schlag P, Tilgen W, Brado B, Galm F, Gorich J, Kauffmann GW, Moller P, Schneider S and Hunstein W: Regional administration of lymphokine-activated killer cells can be superior to intravenous application. Cancer *69*: 2172-2175, 1992.
26 Den Otter W, De Groot JW, Bernsen MR, Heintz APM, Maas R, Hordijk GJ, Hill FWG, Klein WR, Ruitenberg EJ and Rutten VPMG: Optimal regimes for local IL-2 tumour therapy. Int J Cancer *66*: 400-403, 1996.
27 Anderson PM, Katsanis E, Leonard AS, Schow D, Loeffler CM, Goldstein MB and Ochoa AC: Increased local antitumor effects of interleulin 2 liposomes in mice with MCA-106 sarcoma pulmonary metastases. Cancer Res *50*: 1853-1856, 1990.
28 Vaage J: Local interleukin 2 therapy of mouse mammary tumors of various immunogenicites. Cancer Res *48*: 2193-2197, 1988.
29 Moriai T, Makino I and Kikuchi Y: Synergistic antitumor effect of interleukin-2 and irradiation on pancreatic cancer in Syrian golden hamsters. In Vivo *4*: 127-130, 1990.
30 Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D, Robinson BWS and Nelson DJ: IL-2 intratumoral immunotherapy enhances $CD8^+$ T cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for IL-2. J Immunol *171*: 5051-5063, 2003.
31 Dummer R, Becker JC, Boser B, Hartmann AA and Burg G: Successful therapy of metastatic eccrine poroma using perilesional interferon alfa and interleukin 2. Arch Dermatol *128*: 1127-1128, 1992.
32 Gutwald JGJ, Groth W and Mahrle G: Peritumoral injections of interleukin 2 induce tumour regression in metastatic malignant melanoma. Br J Dermatol *130*: 541-542, 1994.
33 Kaplan B and Moy RL: Effect of perilesional injections of PEG-interleukin-2 on basal cell carcinoma. Dermatol Surg *26*: 1037-1040, 2000.

34 Radny P, Caroli UM, Bauer J, Paul T, Schlegel C, Eigentler TK, Weide B, Schwarz M and Garbe C: Phase II trial of intralesional therapy with interleukin-2 in soft-tissue melanoma metastases. Br J Cancer *89*: 1620-1626, 2003.

35 Pockaj BA, Sherry RM, Wei JP, Yannelli JR, Carter CS, Leitman SF, Carasquillo JA, Steinberg SM, Rosenberg SA and Yang JC: Localization of ^{111}indium-labeled tumor infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy. Augmentation with cyclophosphamide and correlation with response. Cancer *73*: 1731-1737, 1994.

36 Sacchi M, Snyderman CH, Heo DS, Johnson JT, d'Amico F, Herberman RB and Whiteside TL: Local adoptive immunotherapy of human head and neck cancer xenografts in nude mice with lymphokine-activated killer cells and interleukin 2. Cancer Res *50*: 3113-3118, 1990.

37 Kjaergaard J, Hokland ME, Agger R, Skovbo A, Nannmark U and Basse PH: Biodistribution and tumor localization of lymphokine-activated killer T cells following different routes of administration into tumor-bearing animals. Cancer Immunol Immunother *48*: 550-560, 2000.

38 Ciatto S, Rosselli Del Turco M, Ambrogetti D, Bravetti P, Catarzi S, Morrone D and Cariaggi MP: Solid nonpalpable breast lesions. Success and failure of guided fine-needle aspiration cytology in a consecutive series of 2444 cases. Acta Radiol *38*: 815-820, 1997.

39 Kamphausen BH, Toellner T and Ruschenburg I: The value of ultrasound-guided fine-needle aspiration cytology of the breast: 354 cases with cytohistological correlation. Anticancer Res *23*: 3009-3014, 2003.

40 Vujanovic NL, Rabinowich H, Lee YT, Jost L, Herberman RB and Whiteside TL: Distinct phenotypic and functional characteristics of human natural killer cells obtained by rapid interleukin 2-induced adherence to plastic. Cell Immunol *151*: 133-157, 1993.

41 Nowak AK, Robinson BWS and Lake RA: Synergy between chemotherapy and immuno-therapy in the treatment of established murine solid tumors. Cancer Res *63*: 4490-4496, 2003.

Received January 9, 2004
Accepted May 6, 2004